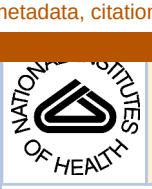
View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Digital.CSIC



NIH Public Access

Author Manuscript

Exp Cell Res. Author manuscript; available in PMC 2006 April 26.

Published in final edited form as:

Exp Cell Res. 2005 August 15; 308(2): 364–380.

Phylogenetic conservation of the regulatory and functional properties of the Vav oncoprotein family

José R. Couceiro[a], **María D. Martín-Bermudo**[b,1], and **Xosé R. Bustelo**[a,*]

a *Centro de Investigación del Cáncer and Instituto de Biología Molecular y Celular del Cáncer, CSIC-University of Salamanca, Campus Unamuno, E-37007 Salamanca, Spain*

b *Instituto de Parasitología y Biomedicina "López-Neyra", CSIC, Ventanilla St. 11, E-18001 Granada, Spain*

Abstract

Vav proteins are phosphorylation-dependent GDP/GTP exchange factors for Rho/Rac GTPases. Despite intense characterization of mammalian Vav proteins both biochemically and genetically, there is little information regarding the conservation of their biological properties in lower organisms. To approach this issue, we have performed a characterization of the regulatory, catalytic, and functional properties of the single Vav family member of *Drosophila melanogaster*. These analyses have shown that the intramolecular mechanisms controlling the enzyme activity of mammalian Vav proteins are already present in *Drosophila*, suggesting that such properties have been set up before the divergence between protostomes and deuterostomes during evolution. We also show that *Drosophila* and mammalian Vav proteins have similar catalytic specificities. As a consequence, *Drosophila* Vav can trigger oncogenic transformation, morphological change, and enhanced cell motility in mammalian cells. *Gain-of-function* studies using transgenic flies support the implication of this protein in cytoskeletal-dependent processes such as embryonic dorsal closure, myoblast fusion, tracheal development, and the migration/guidance of different cell types. These results highlight the important roles of Vav proteins in the signal transduction pathways regulating cytoskeletal dynamics. Moreover, they indicate that the foundations for the regulatory and enzymatic activities of this protein family have been set up very early during evolution.

Keywords

Vav oncoproteins; Rho/Rac GTPases; GDP/GTP exchange factors; Cell migration; Development; Cytoskeleton; *Drosophila*

Introduction

The Vav family is a group of signaling molecules with key roles in cytoskeletal dynamics and oncogenesis [1]. These proteins were discovered initially in mammals and found to be composed of eight structural domains, including a calponin-homology (CH) region, an acidic (Ac) domain, the catalytic Dbl-homology (DH) region, a pleckstrin-homology (PH) domain, a zinc finger (ZF) region, a Src homology (SH) 2 domain, and two SH3 regions (see Supplementary Information, Fig. S1) [1]. Such complex structure is conserved in the three representative members of the Vav family in mammals (Vav, Vav2, and Vav3) [2–4]. The main biological activity of Vav proteins is to act as guanosine nucleotide exchange factors

* Corresponding author. Fax: +34 923 294743. *E-mail address:*xbustelo@usal.es (X.R. Bustelo)..

[1]Present address: Centro Andaluz de Biología del Desarrollo, CSIC-Pablo Olavide University, Carretera de Utrera Km. 1, E-41013 Sevilla, Spain.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

(GEFs) for specific members of the Rho/Rac family [4–6]. This catalytic activity allows the rapid transition of Rho/Rac GTPases from the inactive (GDP-bound) to the active (GTP-bound) state during signal transduction. Vav proteins catalyze nucleotide exchange preferentially on Rac (i.e., Rac1, Rac2, RhoG) and Rho (i.e., RhoA, RhoB) subfamily proteins. In contrast, they are not active on the highly related Cdc42 protein [4–6]. The structural basis for this enzyme selectivity has been described recently for Vav3 [7]. In addition to the activation of Rho/Rac pathways, it has been proposed that Vav proteins activate other signaling responses. Thus, it has been shown that the Vav/Rac1 pathway can promote the activation of the Ras route in lymphocytes via the phospholipase C (PLC)-γ-dependent activation of Ras GDP releasing protein (GRP)1, a Ras-specific GDP/GTP exchange factor whose activity is regulated by the second messenger diacylglycerol [8,9]. Similarly, activation of the Rap pathway via the stimulation of the Rap exchange factor RasGRP2 has also been described [10]. Finally, it has been demonstrated that the C-terminal SH3-SH2-SH3 region mediates the binding to a wide variety of signaling molecules such as Grb2 [11], hnRNP-K [12], Cbl-b [13], and Slp76 [14].

Genetic evidence derived from the use of knockout mice indicates that the function exerted by Vav proteins is crucial for the coordination of developmental and mitogenic processes. Thus, the elimination of the *vav* gene results in impaired lymphoid development, lymphopenia, and defective immune responses in mice [15–19]. Deletion of the *vav2* gene leads to defective signaling responses in activated B-cells [19,20]. The double or triple deletion of *vav*, *vav2*, and/or *vav3* genes results in an accentuated phenotype in some of those responses, indicating that they play partially redundant functions [21]. It has also been demonstrated that the subversion of the normal activation/deactivation cycle of some members of the Vav family results in severe alterations of cell behavior, including tumorigenesis, cell cycle transitions, actin cytoskeleton dynamics, and the acquisition of metastatic properties by transformed cells [1,22].

To avoid those unwanted biological effects, cells have developed a stringent system for regulating the GDP/GTP exchange activity of these proteins. Such control is exerted through the regulation of their catalytic activities by direct phosphorylation on tyrosine residues [1, 23]. Recent structural studies have indicated that this activation step correlates with the relaxation of an autoinhibitory loop established by the interaction of the Vav N-terminal domains (CH and Ac) with other regions of the molecule (DH and ZF) [24,25]. This inhibitory loop limits the access of the GTPase to the catalytic site of the DH domain of Vav proteins, making it impossible the exchange of nucleotides on the GTPase substrates. The inhibitory loop is disrupted upon phosphorylation of a tyrosine residue (Y^{174}) present in the acidic domain, leading to an “open”, catalytically competent conformation of the GEFs [4–6,24]. This balanced physiological regulation is lost when the N-terminal domains of Vav proteins are either eliminated or inactivated by point mutations, leading to the generation of highly oncogenic Vav proteins whose biochemical activities are independent of tyrosine phosphorylation [6,25,26].

The sequencing of genomes from several eukaryotes has allowed the discovery of additional members of the Vav family. We now know that there are single representatives of the Vav family in nematodes (*Caenorhabditis elegans*), insects (*Drosophila melanogaster*, *Anopheles gambiae*), and ascidians (*Ciona intestinalis*). In vertebrates, three family members (Vav, Vav2, and Vav3) have been found in pufferfish, chicken, and mammals. Although less characterized, Vav sequences have been also detected in other fish (*Danio rerio*, *Fundulus heteroclitus*, *Tetraodon nigroviridis*) and amphibia (*Xenopus laevis*, *Xenopus tropicalis*). So far, no Vav family proteins have been found in plants or unicellular eukaryotes such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, or *Plasmodium falciparum*, indicating that this GEF family has probably arisen to fulfill specific functional needs of animal metazoans [1]. All identified Vav proteins display the same structural motifs with the exception of the Vav proteins

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

of nematodes and flies, which lack the most N-terminal SH3 domain (Fig. S1) [1]. Of all these proteins, only the *Drosophila* representative has been studied at the functional level. These analyses have shown that *Drosophila* Vav is a ubiquitous cytosolic protein and a good substrate for membrane tyrosine kinases of the EGF-receptor family [27,28]. These studies have also shown that, at least in tissue culture, *Drosophila* Vav appears to be important for the stimulation of the Ras downstream element ERK [28].

The availability of Vav family proteins from a wide range of species has given us the opportunity to take a look at the evolution of the *vav* loci throughout different species and to get a phylogenetic perspective of the regulation of the Vav family. In this work, we have decided to investigate whether the known regulatory steps of mammalian Vav proteins have been acquired gradually or simultaneously during evolution. To this end, we have focused our attention on the regulatory and functional properties of the Vav protein found in the fly *Drosophila melanogaster*. This protein keeps the most ancestral scaffold of the Vav family and belongs to a species originated after the evolutionary split between protostomes and deuterostomes, thus being an excellent working model to approach issues of regulatory and functional conservation in this protein group. Using both biochemical and genetic strategies, we demonstrate here that all the regulatory controls previously described in human and mice are also found in flies. In addition, we show that *Drosophila* Vav plays key roles in the regulation of the actin cytoskeleton that, when deregulated, give rise to abnormalities in the development of specific tissues.

Materials and methods

Cloning of Drosophila vav cDNA

A 3.0-kb-long full length *Drosophila melanogaster* (*Dm*) *vav* cDNA was obtained by polymerase chain reaction (PCR) amplification from an ovary cDNA library using a custom screening service (Genome Systems) and cloned into pBluescript (Stratagene). Sequencing of the clone confirmed the nature of the cDNA but revealed sequence discrepancies with the previously described cDNA and genomic *Dm vav* clones [27,29,30]. In the case of the available *Dm vav* cDNA clones [27], those changes include single amino acid substitutions (F148L, L372M, R503E, P739Q) as well as replacements of longer protein segments (645-LLRVRPQGPSTAHETMYALS-664 to 645-PVASS-SAGPIHCPRDDVCAY-664). Sequencing of the *Dm vav* cDNA clone obtained from Katzav's laboratory indicated that those disparities were due to sequencing errors in the first cDNA isolate that created either point mutations (changes at positions 148, 373, 503, 739) or frame-shift mutations (the extensive change between residues 645–664). In the case of the available genomic data [29], we found a M53T change as well as a silent change in codon 313. Whether those changes are due to polymorphisms in those areas or to sequence errors in the genomic sequence remains to be determined.

Antibodies

Polyclonal antibodies to the Vav DH domain were raised in rabbits using GST fusion proteins purified from *Escherichia coli*. Anti-Myc, anti-phosphotyrosine, and anti-γ-tubulin antibodies were from Upstate Biotechnology, Santa Cruz Biotechnology, and Sigma, respectively. Anti-HA and AU5 antibodies were obtained from Covance. Fasciclin III, fasciclin II, and anti-22C10 antibodies were obtained from the Developmental Studies of Hybridoma Bank (University of Iowa). The anti-HA antibody used to stain *Drosophila* embryos (clone 3F10) was obtained from Roche Molecular Biochemicals. Secondary antibodies used in immunofluorescence and immunohistochemistry experiments were purchased from Jackson Immunolabs.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Expression vectors and site-directed mutagenesis

For expression studies in mammalian cells, the *Dm vav* cDNA was amplified from the cDNA library isolate and cloned into the pEF1/Myc-HisA plasmid (Invitrogen). After cloning, the *Drosophila* Vav protein became fused in frame to the Myc epitope at the C-terminus. *Drosophila* Vav point mutants were obtained using the QuickChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. Truncated Vav proteins were generated by using either appropriate internal restriction sites according to standard procedures or by PCR using the Elongase polymerase (Invitrogen). For expression in *Drosophila* embryos, *Dm vav* cDNAs were amplified by PCR and cloned into a modified pUAST plasmid encoding a C-terminal HA tag before the termination codon. All cloned and mutant cDNAs were subjected to automatic sequence analysis to avoid the possibility of extra mutations. Details regarding the generation of specific constructs and mutants are available upon request. The wild type and mutated versions of the mouse *vav*, *vav2*, and *vav3* proto-oncogenes have been described before [3,4,6,25,26]. Mammalian *vav* cDNAs used were cloned in pcDNA3 (Invitrogen), pMEX (a homemade plasmid), or pEGFP-C (BD Biosciences Clontech). pCEFL-AU5-Rac1 (wild type), pCEFL-AU5-Rac1^{Q61L}, pCEFL-AU5-RhoA (wild type), pCEFL-AU5-RhoA^{Q63L}, pCEFL-AU5-Cdc42 (wild type), and pCEFL-AU5-Cdc42^{Q61L} plasmids have been described previously [6]. The bacterial expression pGEX vectors encoding the CRIB domains of Wasp and Rhotekin were obtained from Dr. P. Crespo (University of Cantabria/CSIC, Santander, Spain). The pGEX vector containing the Rac1-binding domain of Pak1 was provided by Dr. R. A. Cerione (Cornell University, Ithaca, NY). The GST fusion proteins were expressed in *E. coli* and purified by affinity chromatography onto glutathione-coated beads (Amersham Biosciences) according to standard procedures.

Cell culture and transfections

NIH3T3 and COS1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 1% L-glutamine and 1% penicillin/streptomycin at 37° in an atmosphere of 5% CO_2. All tissue culture reagents were obtained from Invitrogen. For focus formation assays, NIH3T3 cells were transfected using the calcium phosphate precipitation method [31]. One day after transfection, cells were washed with DMEM supplemented with 5% serum and cultured for 15 days with changes of media every 3 days. Cells were then washed with a phosphate-buffered saline solution (PBS), fixed with formaldehyde, and stained with Giemsa. Each transfection was performed in duplicate in at least three independent experiments. For the isolation of stable clones of *Dm*Vav-transformed cells (i.e., JRC2-8), independent foci derived from those transfections were left unstained and picked up with the help of cloning cylinders. Clones were then expanded in culture and frozen according to standard tissue culture protocols. For morphological assays, cells were transfected with liposomes (FuGENE 6, Roche Molecular Biochemicals) according to the manufacturer's recommendations and fixed 48 h later. For protein detection or pull-down experiments, COS1 cells were transfected using the DEAE-dextran (Sigma) method and harvested 48 h later. Wound-healing assays with NIH3T3 cells have been done as described [32].

Immunofluorescence

Cells were cultured on glass coverslips introduced into six-well plates (5×10^5 cells/well). 24 h after transfection, cells were rinsed in PBS and fixed with 3.7% formaldehyde (Sigma) in PBS for 15 min, permeabilized by incubation with PBS containing 0.5% Triton X-100 for 10 min, and blocked in a 25-mM Tris-buffered solution supplemented with 2% bovine serum albumin, 0.1% sodium azide, and 0.1% Triton X-100 for 10 min. Cells were incubated with anti-Myc antibodies (1:400 dilution) for 1 h followed by incubation with FITC-labeled anti-rabbit Ig antibodies for 1 h. Staining of F-actin in fixed cells was done with rhodamine-labeled phalloidin (Molecular Probes), as described [4].

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

GST-pull-down experiments and immunoblots

COS1 cells growing in 10-cm plates were collected 48 h after transfection, washed with ice-cold PBS, and disrupted in a lysis buffer containing 20 mM Tris–HCl [33], 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton-X100, 5 mM β-glycerophosphate, 1 mM DTT, a protease-inhibitor cocktail (*Cømplete*, Roche Molecular Biochemicals), and 10 μg of the appropriate GST fusion protein. Cell lysates were precleared by centrifugation at 11,000 rpm for 10 min at 4°C and then incubated with glutathione-Sepharose beads for 2 h at 4°C. Beads were washed thrice in lysis buffer without GST fusion protein, boiled in SDS-PAGE sample buffer, and the released proteins subjected to Western blot analysis using anti-AU5 antibodies. For protein expression analysis, total cellular lysates were separated electrophoretically and analyzed by immunoblotting according to standard procedures. Immunoreactive protein signals were developed using a chemiluminescent method (ECL, Amersham Biosciences).

Generation of transgenic flies

Constructs were introduced into the germ line using standard methods for P-element transformation and several independent transgenic lines isolated [34]. Targeted expression of UAS-driven transgenes was induced using the following GAL4 lines: *69B*, *24B* [35], *elav* [36], *G447.2* [37], *btl* [38], and *engrailed*-GAL4 (kindly provided by G. Morata). The UAS-$Rac1^{G12V}$ has been described before [36].

Antibody staining and in situ hybridization

Embryos were stained using horseradish peroxidase with biotin-labeled secondary antibodies and the Vectastain Elite ABC kit (Vector Laboratories). In the case of the anti-fasciclin III immunostaining, we used FITC-labeled secondary antibodies. Whole-mount in situ hybridization was done with a *dpp* anti-sense cRNA probe labeled with digoxygenin-UTP (Genius kit, Roche Molecular Biochemicals). Stained embryos were mounted and photographed with a Axiophot microscope (Zeiss) using Nomarski optics. Images were collected digitally and assembled with the Adobe Photoshop software (Adobe Systems Inc).

Results

The regulatory mechanisms controlling the catalytic activity of mammalian Vav proteins are conserved in DmVav

The catalytic activity of mammalian Vav proteins is inhibited in the non-phosphorylated state through a complex system of interactions among four different structural domains. On one hand, the Y^{174} residue of the acidic region interacts with the GTPase binding region of the DH domain [24]. It has been postulated that this action can also be mimicked by two additional tyrosine residues of the acidic domain (Y^{142} and Y^{160}) [24]. On the other hand, the CH region interacts with the ZF region to block completely the access of the GTPase substrates to the catalytic site of Vav proteins [25]. These interactions can be mapped indirectly by assessing the effect of particular mutations on the biological activity of Vav proteins. For instance, Y to F mutations in residues 142, 160, or 174 trigger the activation of the latent transforming activity of mouse full-length Vav protein [26]. In addition, partial (Δ1–66) or total (Δ1–144, Δ1–186) deletions of the Vav CH region generate highly oncogenic mutant proteins [6,25,39]. To investigate whether these two inhibitory mechanisms are present in *Dm*Vav, we analyzed the transforming activity of several *Dm*Vav mutants using focus formation assays in rodent fibroblasts.

We first compared the transforming activity of N-terminal deleted mutants of *Dm*Vav (Δ1–113, Δ1–155, Δ1–207), mouse Vav (Δ1–66, Δ1–144, Δ1–186), and mouse Vav2 (Δ1–66, Δ1–187) (Fig. S1). As previously described [25], the Vav (Δ1–186) and (Δ1–144) mutants

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

displayed very high levels of transforming activity whereas the shorter deletion mutant (Δ1–66) showed a 14- to 18-fold lower oncogenicity (Figs. 1A, B). In the case of Vav2, such activation was only clearly observed with the Δ1–187 mutant (Figs. 1A, B). The number of foci induced by Vav2 is significantly lower than those obtained with analogous Vav mutants (Figs. 1A, B). This is due to a cell cycle arrest induced by Vav2 mutants that originate a high proportion of giant, polynucleated single cells that do not develop further to form foci [3]. The wild type versions of mouse Vav and Vav2 show either very low or no transforming activity (Figs. 1A, B), in good agreement with previous reports [3,6,25,39]. In the same experiments, the progressive deletion of the *Dm*Vav CH-Ac region induces a concomitant increase in the transforming activity of this protein. In fact, the *Dm*Vav (Δ1–207) mutant shows levels of biological activity similar to the analogous Vav (Δ1–186) mutant (Figs. 1A, B). The morphology of *Dm*Vav-derived foci was quite similar to that induced by Vav (Δ1–186) (Fig. 1C, compare the two top panels). In contrast, it was different from cells transformed via the activation of unrelated pathways, such as the RasGRP1/Ras route (Fig. 1C, compare top and lower panels). All *Dm*Vav proteins were similarly expressed in cells, further indicating that the differential transforming activity of *Dm*Vav proteins is a true reflection of their catalytic activities (Fig. 1D, upper panel).

We then tested the transforming activity of *Dm*Vav proteins with Y to F point mutations in positions homologous to the Y^{142} (Y^{165} in *Dm*Vav), Y^{160} (Y^{183} in *Dm*Vav), and Y^{174} (Y^{194} in *Dm*Vav) residues found in mammalian Vav (see Supplementary Information, Fig. S1). As previously described [26], the progressive addition of mutations in this region of mouse Vav leads to increased levels of cellular transformation (Figs. 2A, B). The same phenomenon was observed when those mutations were made in *Dm*Vav (Figs. 2A, B). The Y^{174} residue (Y^{194} in *Dm*Vav) seems to be the main inhibitory residue both in mammalian and *Dm*Vav proteins, since the transforming activity of proteins with mutations in that site is higher than those of Y142F (Y165F in *Dm*Vav) and Y160F (Y183F in *Dm*Vav) single mutants (Figs. 2A, B). All these proteins were expressed at similar levels in cells (Fig. 2C, upper panel). Taken together, these results indicate that the two known regulatory mechanisms for modulating the catalytic activity of mammalian Vav proteins have been set up already in *D. melanogaster*.

Catalytic specificity of DmVav

We next evaluated the level of conservation of the *Dm*Vav catalytic specificity with respect to its mammalian counterparts. To this end, *Dm*Vav (Δ1–1–207) was co-expressed with the wild type versions of Rac1, RhoA, and Cdc42. As a comparative control, these GTPases were co-transfected either alone or with the oncogenic versions of Vav (Δ1–186 mutant), Vav2 (Δ1–187 mutant), or the pan-specific GEF Dbl [40,41]. After the transfections, the activated status of Rac1, RhoA, and Cdc42 was monitored using pull-down experiments with GST proteins fused to the Rho/Rac binding domains of Pak1, Rhotekin, and Wasp, respectively [42]. These proteins bind only to the activated, GTP-bound forms of the appropriate upstream GTPase, thus being adequate tools to determine the activation status of such GTPases in vivo. These experiments revealed that *Dm*Vav (Δ1–207) could activate Rac1 and, at lower levels, RhoA (Figs. 3A–B, left panels, compare first and second lanes). The levels of activation of Rac1 and RhoA were comparable to those induced by mammalian Vav and Vav2 proteins (Figs. 3A–B, left panels). In contrast, *Dm*Vav (Δ1–207) could not activate the highly related Cdc42 protein (Fig. 3C, left panel, compare first and second lanes). This is not due to the inactivity of Cdc42 in our experiments because high levels of GTP-bound Cdc42 were obtained upon coexpression with the *dbl* oncogene (Fig. 3C, left panel, compare first and third lanes). As expected, the constitutively active versions of the GTPases were efficiently pulled down by the respective protein baits (Figs. 3A–D). Immunoblot experiments confirmed that the wild type GTPases were expressed at similar levels in all transfection experiments (Figs. 3A–C, right panels). In agreement with the previous focus formation assays, the activation of Rac1 and RhoA was due

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

to the constitutive activity of the *Dm*Vav (Δ1–207) because wild type *Dm*Vav could not activate Rac1 in vivo despite being expressed at comparable levels to *Dm*Vav (Δ1–207) (Fig. 3D). Taken together, these results indicate that *Dm*Vav has the same catalytic specificity as its related family members from mammals.

DmVav induces F-actin polymerization and cell motility

Since Rho/Rac GTPases have an implication in the organization of the actin cytoskeleton [43,44], we next studied whether *Dm*Vav had some role in regulating the distribution of F-actin in cells. To this end, we first transfected fibroblasts (NIH3T3 cells) with mammalian expression vectors encoding either *Dm*Vav or the *Dm*Vav (Δ1–207) mutant. As a control, we performed similar transfections with vectors encoding EGFP-tagged versions of mammalian Vav (Δ1–186), Vav2 (Δ1–187) or Vav3 (Δ1–144). Twenty-four hours post-transfection, cells were fixed, incubated with rhodamine-labeled phalloidin to visualize the polymerized actin meshwork, and subjected to confocal microscopy analysis. We found that the expression of *Dm*Vav did not induce any detectable morphological change other than the sporadic detection of thin stress fibers in some of the transfected cells (Fig. 4, panel A). Instead, the expression of *Dm*Vav (Δ1–207) led to the radial projection of lamellipodia, extensive ruffling at the periphery of the lamellipodia, and a contractile actomyosin ring. In addition, thick bundles of stress fibers were observed in the central region of *Dm*Vav (Δ1–207)-expressing cells (Fig. 4, panels D and G). We also detected a tendency of *Dm*Vav (Δ1–207)-transfected cells to round up and loose adherence to the substrate (data not shown), an effect probably derived from the tension generated by the actomyosin ring. This effect is frequently observed in cells expressing GTPase deficient versions of RhoA, RhoB, or RhoC [6]. Similar morphological changes were observed with other *Dm*Vav mutants (Δ1–155, Y3xF; data not shown). Instead, the expression of the non-chimeric EGFP did not induce any detectable change in the morphology of the transfected cells (data not shown). The morphological changes induced by *Dm*Vav (Δ1–207) were similar to those induced by the expression of the constitutively active forms of Vav, Vav2, and Vav3 in the same cell setting (Fig. 4, panels J, M, and P, respectively).

We observed a less complex morphological phenotype when *Dm*Vav (Δ1–207) was expressed in COS1 cells since, in this case, the changes were limited to the formation of extensive membrane ruffling both in the periphery and dorsal areas of the transfected cells (Fig. S2A, panel A). Again, this phenotype was similar to that induced by the constitutively active version of mammalian Vav (Fig. S2A, panel D). Similar morphological changes were also observed with *Dm*Vav (Δ1–155, *Dm*Vav (Y3xF), Vav (Y3xF), Vav2 (Δ1–187), and Vav3 (Δ1–144) proteins (data not shown). No morphological changes were seen with either the EGFP alone or the wild type versions of both *Dm*Vav and Vav (data not shown). Taken together, these results indicate that both *Dm*Vav and mammalian Vav can mediate different types of morphological change depending on the cell background used.

To verify whether the activity of *Dm*Vav on the dynamics of the F-actin cytoskeleton resulted in specific enhanced motility responses, we performed wound-healing assays in vitro [32]. In these experiments, a narrow area of a serum-starved cell monolayer is scraped and then left to be refilled by the cells of the two opposing sites of the wound, a response that correlates with the relative motility of the cell population studied. To facilitate these analyses, we used a stable clone of transformed cells (JRC2-8) to make sure that all cells expressed the *Dm*Vav (Δ1–207) protein. We observed that the *Dm*Vav (Δ1–207)-transformed cells healed the scraped areas at much higher paces than the parental, untransformed cells (Fig. S2B, compare upper and lower panels, respectively). Similar results were obtained with Vav (Δ1–186)-transformed cells (data not shown). Taken together, these results indicate that *Dm*Vav induces morphological changes and F-actin dynamics similar to its mammalian counterparts.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Structural requirements for the biological activity of DmVav and mammalian Vav proteins

The above results indicated that the regulatory mechanisms that operate in the N-terminal CH-Ac regions of Vav proteins are evolutionarily conserved. To test whether such functional conservation is a common property of all the structural domains present in Vav proteins, we decided to analyze the biological activity of *Dm*Vav (Δ1–207) proteins with inactivating single point mutations in the DH (L235Q mutation), PH (W532F mutation), ZF (C566S mutation), SH2 (G642V), and SH3 (P779L) domains. These point mutations are located in analogous positions to those previously characterized in the mouse Vav (Δ1–186) oncoprotein [25]. After demonstrating by immunoblot analysis that these mutant proteins were properly expressed in mammalian cells (Fig. 5A), we analyzed their biological activity using both focus formation and cytoskeletal assays in rodent fibroblasts. As control, we included the already described Vav (Δ1–186) mutants for each of those domains [25]. Our focus formation experiments indicated that *Dm*Vav (Δ1–207) cannot elicit cell transformation or cytoskeletal change unless the DH, PH, and ZF regions are functional (Figs. 5B, C; panels D, D′, G, G′, J, and J′, respectively). As expected from previous experiments with mammalian Vav proteins [25], we observed in contrast that the SH2 mutants of *Dm*Vav (Δ1–207) and Vav (Δ1–186) show a reduced, but still high, transforming activity (Fig. 5B). In addition, these two mutants are fully active in the promotion of cytoskeletal change (Fig. 5C, panels M and M′). The biological activity of the SH2 Vav mutants is in good agreement with the phosphorylation-independent exchange activity of the N-terminally deleted oncogenic forms of mammalian and *Drosophila* Vav family proteins [4,6,25] (see also Fig. 3). Taken together, these observations demonstrate that *Drosophila* Vav behaves very similarly to its mouse counterparts in terms of the requirements of DH, PH, ZF, and SH2 domains for optimal biological activity.

In contrast to these results, we observed that *Dm*Vav (Δ1–207), unlike mouse Vav (Δ1–186) [25], is highly dependent on the C-terminal SH3 region for optimal biological activity. Thus, mouse Vav can induce cytoskeletal change when mutated in the SH3 region or, alternatively, upon deletion of the entire SH3-SH2-SH3 region (Fig. 5C, panels P and S, respectively) [25]. In contrast, we observed that *Dm*Vav (Δ1–207) cannot trigger cytoskeletal change when mutated in the SH3 region (Fig. 5C, panel P′). This differential behavior was also observed in focus formation assays. Thus, Vav (Δ1–186) and its SH3 mutant show identical transforming activity when tested in focus formation assays (Fig. 5B) [25]. In contrast, the transforming activity of *Dm*Vav (Δ1–207) is totally lost upon mutation of the SH3 region (Fig. 5B). The differential dependency on the SH3 domain of *Dm*Vav and mouse Vav is not due to the presence of an additional SH3 region in the mouse oncoprotein (see Fig. S1), since mouse mutant proteins lacking both the CH-Ac and the entire SH3-SH2-SH3 region can still induce high levels of cell transformation [6,25]. Taken together, these results indicate the regulatory function of the SH3 region is not conserved phylogenetically in the Vav family. This is a biological property unique to *Dm*Vav, since the three known mammalian Vav family members can trigger cytoskeletal change and/or cell transformation regardless of the functionality of their SH3 domains [4,6,25]. In contrast, the rest of structural domains of Vav proteins (CH, Ac, DH, PH, ZF, and SH2 domains) behave very similarly in flies and mammals.

Developmental defects caused by the deregulated activity of DmVav in Drosophila embryos

To get further insights on the function of *Dm*Vav in vivo, we finally analyzed the effect of this protein in *Drosophila* development using a *gain-of-function* approach. To this end, we generated an extensive collection of fly strains that expressed either wild type *DmVav* or *Dm*Vav (Δ1–207) proteins in specific tissues of the embryo via the use of the GAL4 promoter system [35]. In these studies, we focused our interest on a number of developmental programs highly dependent on cytoskeletal dynamics, including the processes of dorsal closure, myoblast fusion, nervous system architecture, migration of cells of the caudal visceral mesoderm, and tracheal development.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

During *Drosophila* development, two opposing layers of epidermal sheets move towards each other, meeting and fusing seamlessly at the dorsal midline. This process is believed to be regulated by an actomyosin contractile ring that assembles at the leading edge of the approaching epidermal sheets, with lamellipodial and filopodial protusions facilitating the subsequent adhesion and alignment during the fusion process [45–48]. When *Dm*Vav (Δ1–207) was expressed in epidermis from stage 9 using the 69B-GAL4 line [35], we observed that it caused defects in dorsal closure very similar to those obtained upon *Dm*Rac1 activation [49,50] (Fig. 6A). To look at the epidermal cell changes occurring during this process, we stained the embryos with antibodies to fasciclin III [51], a glycoprotein expressed on all epidermal cell surfaces except the dorsal ends of the cells flanking the amnioserosa (Fig. 6B, panels A and C). Examination of the lateral epidermis of *Dm*Vav (Δ1–207)-expressing embryos indicated that cells were disorganized and did not show proper elongation. In addition, most cells flanking the dorsal hole showed an abnormal localization of fasciclin III on their dorsal sides (Fig. 6B, panels B and D). The embryos expressing wild type *Dm*Vav were indistinguishable from the wild type ones both in terms of dorsal closure and fasciclin III localization (data not shown).

Although poorly understood, cytoskeletal changes are also important during the fusion of myoblasts to form the multinucleated fibers of the *Drosophila* muscle. It is speculated that this process involves the formation of a vesicular prefusion complex that assembles at the apposed plasma membranes [52,53]. We investigated the action of *Dm*Vav proteins in this process by expressing them under the control of a mesoderm (24B) line [54]. Overexpression of wild type *Dm*Vav did not result in any detectable developmental defect in muscle, as determined by staining of muscle fibers with an anti-muscle myosin antibodies (data not shown). The expression of *Dm*Vav (Δ1–207) resulted in an inhibition of myoblast fusion throughout the somatic mesoderm (Fig. 7, compare B and A panels). This phenotype closely resembles that obtained upon expression of a constitutively activated form (G12V mutant) of *Dm*Rac1 [36].

The most complex changes in cell shape during the development of *Drosophila* probably occur in the nervous system. During this process, the differentiating neurons extend axons and dendrites towards their specific target cells in a GTPase-dependent manner [36,50,55,56]. To analyze the consequences of the constitutive activation of the *Dm*Vav pathways in the development of the nervous system, we examined the embryonic central (CNS) and peripheral (PNS) nervous systems in embryos expressing *Dm*Vav proteins under the control of the nervous system-specific GAL4 line *elav*. *elav*-Gal4 expresses the transgenes in all neurons in embryos starting at stage 12 [36]. To visualize the CNS axon pathways, we used an antibody against fasciclin II that labels axons in three longitudinal fascicles on each side of the midline [57]. We observed several axon guidance defects such as misrouting of some longitudinal axons across the midline and gaps between segments in *Dm*Vav (Δ1–207)-expressing embryos (Fig. 7, panel D, asterisk). In addition, axons looked in general thicker than in wild type embryos (Fig. 7, panel D, arrow). The CNS was absolutely normal in lines expressing wild type *Dm*Vav (data not shown). To visualize the architecture of the PNS, we used the antibody 22C10 that labels the cell bodies, dendrites, and axons of all PNS neurons [58]. Each segment of wild type and *Dm*Vav-expressing embryos contains the highly stereotyped dorsal, lateral, and ventral clusters of PNS neurons connected by axon bundles coming from the dorsal clusters (Fig. 7, panel E and data not shown). In contrast, such axonal connections are missing in most segments of *Dm*Vav (Δ1–207)-expressing embryos (Fig. 7, panels E, F; arrows). Since this fly line expresses the transgene after axon sprouting [36], these results suggest that the phenotype observed in *Dm*Vav (Δ1–207)-expressing embryo is due to problems in axon elongation rather than initiation. These defects are remarkably similar to those observed upon constitutive activation of *Dm*Rac1 in the nervous system [36].

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Another biological process that is highly dependent on actin dynamics is the migration of cells of the caudal visceral mesoderm (CVM). These cells originate in the posterior region of the mesoderm and then migrate in an orderly movement towards the anterior pole of the embryo to form the outer layer of longitudinal muscle fibers that surround the midgut [59]. The influence of *Dm*Vav in this process was assessed by expressing its wild type and oncogenic versions in CMV cells using the GAL4–G447.2 driver line [37]. The localization of CMV cells was visualized using antibodies to either CD2 (expressed by the driver promoter) or HA (present at the C-termini of *Dm*Vav proteins). CMV cells expressing the wild type version of *Dm*Vav showed the expected migration pattern towards the anterior pole of the embryo (Fig. 8, panel A). However, the active mutants of *Dm*Rac1 and *Dm*Vav totally blocked such migration (Fig. 8, panels B and C, respectively). Despite these migratory defects, the CMV cells remained viable at the posterior end of the embryo, indicating that the hyperactivation of the Rac pathway under these two conditions does not influence cell survival or proliferation.

The tracheal system is derived from segmentally repeated epithelial cell clusters of approximately 80 ectodermal cells that undergo branching and migration processes to form a network of tubular epithelia. While the primary branches are extended toward target sites, cell rearrangement takes place to convert these branches into thin unicellular tubules consisting of cells with autocellular junctions. Because these processes take place without cell division, cell-shape changes and cell rearrangement play major roles in the formation of the tracheal network [60–62]. To assess the effects of *Dm*Vav in this developmental process, we expressed *Dm*Vav and *Dm*Vav (Δ1–207) in the tracheal system by using the previously described GAL4-*btl* line [38]. As a control, we included in our analysis embryos expressing *Dm*Rac1^{G12V}. The tracheal phenotypes in each genetic condition were then examined by using a monoclonal antibody that recognizes the tracheal lumen (mAb2A12) [63]. While *Dm*Vav had no effect in tracheal development (data not shown), the expression of either *Dm*Vav (Δ1–207) or *Dm*Rac1^{G12V} induced a variety of defects in tracheal cell migration and differentiation. In the case of *Dm*Vav (Δ1–207)-expressing embryos, they displayed a severe truncation of the tracheal dorsal trunk, misguided dorsal branching, and very limited terminal branching differentiation (Fig. 8, compare panel F with D). The phenotype of *Dm*Rac1^{G12V}-expressing embryo was slightly milder, since the dorsal trunk could get formed in some segments but not in others (Fig. 8, compare panel E with D). However, these embryos showed severe defects in dorsal branching (Fig. 8, panel E).

DmVav cannot connect Rac1 activation to JNK stimulation in Drosophila embryonic epidermal cells

The above results, together with the previous single cell assays for morphological change, are consistent with the idea that *Dm*Vav plays active roles in the Rac1 pathways directly linked to the regulation of F-actin dynamics. To verify whether *Dm*Vav could also trigger other Rac1 responses, we determined whether *Dm*Vav (Δ1–207) could activate the c-Jun N-terminal kinase (JNK), a serine/threonine kinase that works as a Rac1 downstream element in some signaling pathways [44]. To this end, we expressed *Dm*Vav (Δ1–207) in segmental stripes of the ectoderm using the *engrailed*-GAL4 line and checked the activation of *decapentaplegic* (*dpp*), a well-known JNK gene target [64]. While *Dm*Rac1^{G12V} was able to induce ectopic *dpp* expression in the ectoderm [64], *Dm*Vav (Δ1–207) could not (Fig. 9, panels B and C, respectively). Thus, it seems that *Dm*Vav can condition the type of effectors that can be stimulated by the GTP-bound GTPase.

Discussion

Rho/Rac GTPase pathways originated in yeast to regulate functions related to stress responses and F-actin dynamics. Since then, they have adapted to the new functional needs of more

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

complex organisms, such as embryonic development, the maintenance of physiological circuits, or the engagement of immune responses [43,44,65]. This has led to the development of signaling elements that allowed the insertion of these GTPases into new biological pathways. A good example for this progressive acquisition of signaling elements is the Vav oncoprotein family, a group of Rho/Rac GEFs of animal metazoans that have originated to facilitate the connection of Rho/Rac proteins to receptors with intrinsic or associated tyrosine kinase activity [1]. The evolution of these proteins was progressive, both in terms of total gene family number and protein domain structure. Thus, the Vav family has single representatives in protostomes and early chordates but, upon genome duplication events occurring during evolution, diversified later on to give rise to the three known Vav proteins of vertebrates (Vav, Vav2, and Vav3) [1]. Vav proteins acquired new structural features during those transitions, such as the insertion of a proline rich region (missing in the Vav protein from *C. elegans*) and an additional SH3 domain (missing in the Vav proteins of all protostome species). In addition, upon the triplication of the ancestral *vav* gene, they diversified functionally. As a consequence, the three mammalian Vav proteins share a core of basic pathways (i.e., activation of GTPases, modulation of F-actin dynamics) but differ in their ability to engage other signaling responses (i.e., the activation of the nuclear factor of stimulated T-cells) [1,66,67].

The availability of Vav family proteins from a wide range of species has given us the opportunity to take a phylogenetic perspective of the regulation and function of these proteins. In this regard, our characterization of the single Vav family protein of *Drosophila* indicates that the regulatory mechanisms controlling the catalytic activity of its mammalian counterparts have been set up early in evolution. Using a mutagenesis approach, we could demonstrate that the two known structural interactions for regulating the phosphorylation-dependent catalytic activity of Vav proteins are also at work in *Drosophila*. Moreover, we have observed that *Dm*Vav activates the same spectrum of GTPases as mammalian Vav. As a consequence, *Dm*Vav induces biological responses quite similar to its mammalian counterparts when expressed in mammalian cells, including oncogenesis, changes in the cell cytoskeleton, and enhanced cell motility. These results indicate that the foundations for the regulatory and catalytic properties of this protein group were established before the split between protostomes and deuterostomes.

We have also observed that the similarity of the regulatory properties of *Dm*Vav and mouse Vav protein can be extended to most of the other structural domains. On one hand, we have shown that the mutation of key residues of the DH, PH, and ZF region results in the total abrogation of the biological activity of all Vav proteins tested, both in terms of transforming activity and cytoskeletal change. On the other hand, we have demonstrated that the SH2 regions do not play an essential role in the biological activity of Vav oncoproteins. This is probably due to the fact that the N-terminally deleted oncoproteins show a constitutive, phosphorylation-independent exchange activity [4,6,25]. Due to this, they do not rely necessarily in the imperative interaction with upstream tyrosine kinases for activation. This is in agreement with the extensive work with mammalian Vav proteins indicating that the SH2 domains are only essential for the activity of the wild type forms of these exchange factors [4,6,25]. In this regard, the lower transformation observed in the SH2 mutants has been attributed not to lack of phosphorylation but, rather, to a deficient translocation to the plasma membrane [25]. Indeed, if such defect is bypassed by the attachment of membrane localization signals to the Vav C-terminus, the DH-PH-ZF domains of mammalian Vav proteins show even higher transforming activities than the normal, N-terminal deleted oncoproteins that contain the SH3-SH2-SH3 cassette [25].

Unexpectedly, our mutagenesis experiments have revealed that such functional conservation cannot be extended to the SH3 regions. Thus, unlike mammalian Vav proteins [25], *Dm*Vav does not elicit cytoskeletal change when its SH3 region is inactivated by point mutation.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Likewise, the transforming activity of this mutant is also severely reduced. This differential effect cannot be attributed to the presence of a second SH3 region in mammalian Vav proteins, because mouse Vav proteins lacking both SH3 regions can still promote cell transformation and cytoskeletal change [4,6,25]. Despite intense efforts aimed at characterizing the function of the SH3 regions of mammalian Vav proteins, their specific role within the cell remains still obscure. On one hand, it has been shown that this SH3 can bind to a number of proline-rich region containing proteins such as hnRNP-K, Cbl-b and zyxin. On the other hand, it has been postulated that it plays a role in ensuring the proper and efficient subcellular localization of the protein since, as indicated above, its missing function can be fully replaced by the introduction of ectopic membrane localization signals at the C-terminus of the Vav ZF region [1,22,25]. It is likely that this last function could be conserved in *Dm*Vav, because its SH3 mutant cannot be ever detected at the plasma membrane (see Fig. 5C, panel Q′). In any case, these results suggest that, in contrast to the CH, Ac, DH, PH, ZF, and SH2 regions, the regulatory plan for the SH3 regions (both in terms of number of domains and function) have been set up after the protostome/deuterostome split. In this regard, it must be recalled that the most N-terminal SH3 region of Vav proteins has been acquired at the level of *C. intestinalis*, a urochordate species that is considered the most immediate ancestor of the vertebrate lineage.

While our biochemical and tissue culture experiments have pin-pointed the connection of *Dm*Vav with Rac1 and F-actin dynamics, the *gain-of-function* studies carried out in *Drosophila* embryos has given us the opportunity to check the effect of the catalytic activity of *Dm*Vav in a more physiological context. Using transgenic flies expressing the constitutively active form of *Dm*Vav in specific tissues of the *Drosophila* embryo, we could demonstrate that the ectopic activation of this GEF results in developmental problems remarkably similar to those previously observed for Rac1 mutants. Those included defects in embryonic dorsal closure, myoblast fusion, axon growth and guidance, tracheal cell development, and the migration of different populations of cells. The similarity of phenotypes is consistent with the idea that *Dm*Vav and *Dm*Rac1 act in common pathways. These results are probably a reflection of the actual role of *Dm*Vav in those cells, since this protein has a ubiquitous expression in most of the tissues used in our studies [27](our own unpublished observations).

Interestingly, our genetic studies have also indicated that *Dm*Vav may not be able of inducing the activation of all the specific downstream elements of the Rac1 route, as evidenced by the lack of proper activation of the JNK–Dpp pathway in specific cells of *Drosophila* embryos. Although these observations may seem counterintuitive in principle, recent results have shown that it is not a rare signaling event in GEF/Rho–Rac GTPases relationships. For instance, *Dm*Trio, a Rac1-specific GEF widely expressed in *Drosophila*, is only required for Rac1 function in axon growth and guidance but not for epithelial morphogenesis or myoblasts fusion [68]. In mammalian cells, Bokoch and colleagues have shown that the FGD1 GEF triggers JNK activation while having no effect on Pak1 [69]. Conversely, the GEFs Tiam1 and Dbl induce the activation of Pak1 but not of JNK [69]. There are several functional scenarios to explain such signaling selectivity. It can be argued that GEFs may act at subcellular localizations that can be fully compatible with Rac1 activation but not accessible to specific downstream elements. However, experimental evidence does not support this possibility, since the subcellular localizations of Trio, Tiam1, and Dbl are very similar, at least when their oncogenic variants are expressed in mammalian cells (unpublished observations). It is also possible that the stimulation of specific signaling pathways may require the presence of intermediary adaptors recruited by the GEF that facilitate the physical proximity between the GTP-bound GTPase and the primary effector. If that is the case, the specificity of the effector molecules would be determined by the spectrum of adaptor molecules that the GEF can bind to. This possibility has been confirmed already for some GEFs for the Rho/Rac family. Thus, it has been reported that the N-terminal region of the Tiam1 GEF can bind to either spinophilin or IB2/JIP2, two proteins that facilitate the connection of the activated GTPase with $p70^{S6}$ and

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

p38 kinases, respectively [70,71]. Moreover, it has been postulated that the effective activation of Pak1 by Rac1 during T-cell signaling requires the simultaneous association of Vav with Rac1 and Nck, an adaptor protein that can bind to that Rac1 effector [72]. Further genetic and biochemical work in this area will be needed to elucidate the group of Rac1 effectors stimulated by *Dm*Vav and the basis for such signaling specificity. Based on these results, it will also be interesting to use cells from the available *vav*, *vav2*, and *vav3* knockout mice to check the Rho/Rac downstream elements that are specifically affected by the catalytic activity of Vav proteins.

Our observation that the catalytic regulation of the Vav family has been established before the split between protostomes and deuterostomes poises interesting questions regarding the evolutionary time-point at which such functional plan may have been set up originally. Based on previous sequencing data from unicellular and multi-cellular organisms, it was assumed that Vav proteins were totally restricted to animal metazoans. However, a recent report has indicated the presence of tyrosine kinase-related pathways in choanoflagellates (i.e., *Monosiga brevicollis*) [73], a group of unicellular and colonial flagellates that resemble cells found only in metazoa [74]. Recent characterization of EST clones from those protozoa resulted in the isolation of five tyrosine kinases distantly related to the Src/Abl, Tie/Tec, and the FGF-receptor families [73]. More importantly to our case, they appear to express also *vav*-related cDNA sequences [73]. Thus, the ancestor for *vav* family genes could be located much earlier in the phylogenetic tree than previously anticipated. If this is the case, the isolation of this distant family ancestor will be an invaluable tool to track down the molecular evolution of this group of signal transduction molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors wish to thank M. Blázquez and A. Fernández-Ibáñez for technical assistance. We also thank Dr. S. Katzav (The Hebrew University-Hadassah Medical School, Jerusalem, Israel) for making available to us her *Dm vav* cDNA, Dr. A. González-Reyes (Centro Andaluz de Biología del Desarrollo, Sevilla, Spain) for his help in the initial steps of the transgenic fly work, and Drs. M. Dosil and F. Núñez for comments on the manuscript. This work was supported by the US National Cancer Institute (5RO1-CA73735-08 to XRB), the Association for International Cancer Research (00-061 to XRB), the Biomedicine Program of the Spanish Ministry of Education and Science (SAF2003-00028 and BMC2001-2298 to XRB and MDM-B, respectively), and a grant from the Ministry of Education and Culture of the Autonomous Government of Castilla-León (SA051/02 to XRB). J.R.C. is a student of the Molecular and Cellular Cancer Biology graduate program of the CIC and the University of Salamanca who is supported by a FPI fellowship (FP2000-6489) of the Spanish Ministry of Education and Science. M.D.M-B. is a Young Investigator of EMBO. The Centro de Investigación del Cáncer is supported by endowments from the CSIC, University of Salamanca, Castilla-León Autonomous Government, the Spanish Cooperative Network of Cancer Centers (C03/10, Spanish Ministry of Health), and the Foundation for Cancer Research of Salamanca (FICUS).

References

1. Bustelo XR. Regulatory and signaling properties of the Vav family. Mol Cell Biol 2000;20:1461–1477. [PubMed: 10669724]
2. Katzav S, Martin-Zanca D, Barbacid M. Vav, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells. EMBO J 1989;8:2283–2290. [PubMed: 2477241]
3. Schuebel KE, Bustelo XR, Nielsen DA, Song BJ, Barbacid M, Goldman D, Lee IJ. Isolation and characterization of murine vav2, a member of the vav family of proto-oncogenes. Oncogene 1996;13:363–371. [PubMed: 8710375]
4. Movilla N, Bustelo XR. Biological and regulatory properties of Vav-3, a new member of the Vav family of oncoproteins. Mol Cell Biol 1999;19:7870–7885. [PubMed: 10523675]
5. Crespo P, Schuebel KE, Ostrom AA, Gutkind JS, Bustelo XR. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. Nature 1997;385:169–172. [PubMed: 8990121]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

6. Schuebel KE, Movilla N, Rosa JL, Bustelo XR. Phosphorylation-dependent and constitutive activation of Rho proteins by wild-type and oncogenic Vav-2. EMBO J 1998;17:6608–6621. [PubMed: 9822605]
7. Movilla N, Dosil M, Zheng Y, Bustelo XR. How Vav proteins discriminate the GTPases Rac1 and RhoA from Cdc42. Oncogene 2001;20:8057–8065. [PubMed: 11781818]
8. Caloca MJ, Zugaza JL, Matallanas D, Crespo P, Bustelo XR. Vav mediates Ras stimulation by direct activation of the GDP/GTP exchange factor Ras GRP1. EMBO J 2003;22:3326–3336. [PubMed: 12839994]
9. Zugaza JL, Caloca MJ, Bustelo XR. Inverted signaling hierarchy between RAS and RAC in T-lymphocytes. Oncogene 2004;23:5823–5833. [PubMed: 15184873]
10. Caloca MJ, Zugaza JL, Vicente-Manzanares M, Sanchez-Madrid F, Bustelo XR. F-actin-dependent translocation of the Rap1 GDP/GTP exchange factor RasGRP2. J Biol Chem 2004;279:20435–20446. [PubMed: 14988412]
11. Ye ZS, Baltimore D. Binding of Vav to Grb2 through dimerization of Src homology 3 domains. Proc Natl Acad Sci U S A 1994;91:12629–12633. [PubMed: 7809090]
12. Bustelo XR, Suen KL, Michael WM, Dreyfuss G, Barbacid M. Association of the vav proto-oncogene product with poly(rC)-specific RNA-binding proteins. Mol Cell Biol 1995;15:1324–1332. [PubMed: 7862126]
13. Bustelo XR, Crespo P, Lopez-Barahona M, Gutkind JS, Barbacid M. Cbl-b, a member of the Sli-1/c-Cbl protein family, inhibits Vav-mediated c-Jun N-terminal kinase activation. Oncogene 1997;15:2511–2520. [PubMed: 9399639]
14. Wu J, Motto DG, Koretzky GA, Weiss A. Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. Immunity 1996;4:593–602. [PubMed: 8673706]
15. Wulfing C, Bauch A, Crabtree GR, Davis MM. The vav exchange factor is an essential regulator in actin-dependent receptor translocation to the lymphocyte-antigen-presenting cell interface. Proc Natl Acad Sci U S A 2000;97:10150–10155. [PubMed: 10963677]
16. Turner M, Mee PJ, Walters AE, Quinn ME, Mellor AL, Zamoyska R, Tybulewicz VL. A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. Immunity 1997;7:451–460. [PubMed: 9354466]
17. Reynolds LF, Smyth LA, Norton T, Freshney N, Downward J, Kioussis D, Tybulewicz VL. Vav1 transduces T cell receptor signals to the activation of phospholipase C-gamma1 via phosphoinositide 3-kinase-dependent and-independent pathways. J Exp Med 2002;195:1103–1114. [PubMed: 11994416]
18. Zhang R, Alt FW, Davidson L, Orkin SH, Swat W. Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene. Nature 1995;374:470–473. [PubMed: 7700359]
19. Turner M, Billadeau DD. VAV proteins as signal integrators for multi-subunit immune-recognition receptors. Nat Rev, Immunol 2002;2:476–486. [PubMed: 12094222]
20. Doody GM, Bell SE, Vigorito E, Clayton E, McAdam S, Tooze R, Fernandez C, Lee IJ, Turner M. Signal transduction through Vav-2 participates in humoral immune responses and B cell maturation. Nat Immunol 2001;2:542–547. [PubMed: 11376342]
21. Fujikawa K, Miletic AV, Alt FW, Faccio R, Brown T, Hoog J, Fredericks J, Nishi S, Mildiner S, Moores SL, Brugge J, Rosen FS, Swat W. Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells. J Exp Med 2003;198:1595–1608. [PubMed: 14623913]
22. Bustelo XR. Vav proteins, adaptors and cell signaling. Oncogene 2001;20:6372–6381. [PubMed: 11607839]
23. Bustelo XR. Regulation of Vav proteins by intramolecular events. Front Biosci 2002;7:d24–d30. [PubMed: 11779690]
24. Aghazadeh B, Lowry WE, Huang XY, Rosen MK. Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation. Cell 2000;102:625–633. [PubMed: 11007481]
25. Zugaza JL, Lopez-Lago MA, Caloca MJ, Dosil M, Movilla N, Bustelo XR. Structural determinants for the biological activity of Vav proteins. J Biol Chem 2002;277:45377–45392. [PubMed: 12228230]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

26. Lopez-Lago M, Lee H, Cruz C, Movilla N, Bustelo XR. Tyrosine phosphorylation mediates both activation and downmodulation of the biological activity of Vav. Mol Cell Biol 2000;20:1678–1691. [PubMed: 10669745]
27. Dekel I, Russek N, Jones T, Mortin MA, Katzav S. Identification of the *Drosophila melanogaster* homologue of the mammalian signal transducer protein. Vav, FEBS Lett 2000;472:99–104.
28. Hornstein I, Mortin MA, Katzav S. DroVav, the *Drosophila melanogaster* homologue of the mammalian Vav proteins, serves as a signal transducer protein in the Rac and DER pathways. Oncogene 2003;22:6774–6784. [PubMed: 14555990]
29. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richards S, Ashburner M, Henderson SN, Sutton GG, Wortman JR, Yandell MD, Zhang Q, Chen LX, Brandon RC, Rogers YH, Blazej RG, Champe M, Pfeiffer BD, Wan KH, Doyle C, Baxter EG, Helt G, Nelson CR, Gabor GL, Abril JF, Agbayani A, An HJ, Andrews-Pfannkoch C, Baldwin D, Ballew RM, Basu A, Baxendale J, Bayraktaroglu L, Beasley EM, Beeson KY, Benos PV, Berman BP, Bhandari D, Bolshakov S, Borkova D, Botchan MR, Bouck J, Brokstein P, Brottier P, Burtis KC, Busam DA, Butler H, Cadieu E, Center A, Chandra I, Cherry JM, Cawley S, Dahlke C, Davenport LB, Davies P, de Pablos B, Delcher A, Deng Z, Mays AD, Dew I, Dietz SM, Dodson K, Doup LE, Downes M, Dugan-Rocha S, Dunkov BC, Dunn P, Durbin KJ, Evangelista CC, Ferraz C, Ferriera S, Fleischmann W, Fosler C, Gabrielian AE, Garg NS, Gelbart WM, Glasser K, Glodek A, Gong F, Gorrell JH, Gu Z, Guan P, Harris M, Harris NL, Harvey D, Heiman TJ, Hernandez JR, Houck J, Hostin D, Houston KA, Howland TJ, Wei MH, Ibegwam C, Jalali M, Kalush F, Karpen GH, Ke Z, Kennison JA, Ketchum KA, Kimmel BE, Kodira CD, Kraft C, Kravitz S, Kulp D, Lai Z, Lasko P, Lei Y, Levitsky AA, Li J, Li Z, Liang Y, Lin X, Liu X, Mattei B, McIntosh TC, McLeod MP, McPherson D, Merkulov G, Milshina NV, Mobarry C, Morris J, Moshrefi A, Mount SM, Moy M, Murphy B, Murphy L, Muzny DM, Nelson DL, Nelson DR, Nelson KA, Nixon K, Nusskern DR, Pacleb JM, Palazzolo M, Pittman GS, Pan S, Pollard J, Puri V, Reese MG, Reinert K, Remington K, Saunders RD, Scheeler F, Shen H, Shue BC, Siden-Kiamos I, Simpson M, Skupski MP, Smith T, Spier E, Spradling AC, Stapleton M, Strong R, Sun E, Svirskas R, Tector C, Turner R, Venter E, Wang AH, Wang X, Wang ZY, Wassarman DA, Weinstock GM, Weissenbach J, Woodage T, Worley KC, Wu D, Yang S, Yao QA, Ye J, Yeh RF, Zaveri JS, Zhan M, Zhang G, Zhao Q, Zheng L, Zheng XH, Zhong FN, Zhong W, Zhou X, Zhu S, Zhu X, Smith HO, Gibbs RA, Myers EW, Rubin GM, Venter JC. The genome sequence of *Drosophila melanogaster*. Science 2000;287:2185–2195. [PubMed: 10731132]
30. Myers EW, Sutton GG, Delcher AL, Dew IM, Fasulo DP, Flanigan MJ, Kravitz SA, Mobarry CM, Reinert KH, Remington KA, Anson EL, Bolanos RA, Chou HH, Jordan CM, Halpern AL, Lonardi S, Beasley EM, Brandon RC, Chen L, Dunn PJ, Lai Z, Liang Y, Nusskern DR, Zhan M, Zhang Q, Zheng X, Rubin GM, Adams MD, Venter JC. A whole-genome assembly of *Drosophila*. Science 2000;287:2196–2204. [PubMed: 10731133]
31. van der Eb AJ, Graham FL. Assay of transforming activity of tumor virus DNA. Methods Enzymol 1980;65:826–839. [PubMed: 6246378]
32. Kulkarni SV, Gish G, van der Geer P, Henkemeyer M, Pawson T. Role of p120 Ras-GAP in directed cell movement. J Cell Biol 2000;149:457–470. [PubMed: 10769036]
33. Keller G, Kennedy M, Papayannopoulou T, Wiles MV. Hematopoietic commitment during embryonic stem cell differentiation in culture. Mol Cell Biol 1993;13:473–486. [PubMed: 8417345]
34. Spradling AC, Rubin GM. Transposition of cloned P elements into *Drosophila* germ line chromosomes. Science 1982;218:341–347. [PubMed: 6289435]
35. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 1993;118:401–405. [PubMed: 8223268]
36. Luo L, Liao YJ, Jan LY, Jan YN. Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev 1994;8:1787–1802. [PubMed: 7958857]
37. Georgias C, Wasser M, Hinz U. A basic-helix–loop–helix protein expressed in precursors of *Drosophila* longitudinal visceral muscles. Mech Dev 1997;69:115–124. [PubMed: 9486535]
38. Sato M, Kornberg TB. FGF is an essential mitogen and chemo-attractant for the air sacs of the *Drosophila* tracheal system. Dev Cell 2002;3:195–207. [PubMed: 12194851]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

39. Coppola J, Bryant S, Koda T, Conway D, Barbacid M. Mechanism of activation of the vav protooncogene. Cell Growth Differ 1991;2:95–105. [PubMed: 2069873]
40. Hart MJ, Eva A, Zangrilli D, Aaronson SA, Evans T, Cerione RA, Zheng Y. Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. J Biol Chem 1994;269:62–65. [PubMed: 8276860]
41. Hart MJ, Eva A, Evans T, Aaronson SA, Cerione RA. Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product. Nature 1991;354:311–314. [PubMed: 1956381]
42. van Triest M, Bos JL. Pull-down assays for guanoside 5′-triphosphate-bound Ras-like guanosine 5′-triphosphatases. Methods Mol Biol 2004;250:97–102. [PubMed: 14755082]
43. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature 2002;420:629–635. [PubMed: 12478284]
44. Van Aelst L, D'Souza-Schorey C. Rho GTPases and signaling networks. Genes Dev 1997;11:2295–2322. [PubMed: 9308960]
45. Young PE, Richman AM, Ketchum AS, Kiehart DP. Morphogenesis in *Drosophila* requires nonmuscle myosin heavy chain function. Genes Dev 1993;7:29–41. [PubMed: 8422986]
46. Edwards KA, Demsky M, Montague RA, Weymouth N, Kiehart DP. GFP-moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in *Drosophila*. Dev Biol 1997;191:103–117. [PubMed: 9356175]
47. Jacinto A, Wood W, Balayo T, Turmaine M, Martinez-Arias A, Martin P. Dynamic actin-based epithelial adhesion and cell matching during *Drosophila* dorsal closure. Curr Biol 2000;10:1420–1426. [PubMed: 11102803]
48. Jacinto A, Wood W, Woolner S, Hiley C, Turner L, Wilson C, Martinez-Arias A, Martin P. Dynamic analysis of actin cable function during *Drosophila* dorsal closure. Curr Biol 2002;12:1245–1250. [PubMed: 12176336]
49. Harden N, Loh HY, Chia W, Lim L. A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*. Development 1995;121:903–914. [PubMed: 7720592]
50. Hakeda-Suzuki S, Ng J, Tzu J, Dietzl G, Sun Y, Harms M, Nardine T, Luo L, Dickson BJ. Rac function and regulation during *Drosophila* development. Nature 2002;416:438–442. [PubMed: 11919634]
51. Patel NH, Snow PM, Goodman CS. Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. Cell 1987;48:975–988. [PubMed: 3548998]
52. Wakelam MJ. The fusion of myoblasts. Biochem J 1985;228:1–12. [PubMed: 3890835]
53. Doberstein SK, Fetter RD, Mehta AY, Goodman CS. Genetic analysis of myoblast fusion: blown fuse is required for progression beyond the prefusion complex. J Cell Biol 1997;136:1249–1261. [PubMed: 9087441]
54. Martin-Bermudo MD, Dunin-Borkowski OM, Brown NH. Specificity of PS integrin function during embryogenesis resides in the alpha subunit extracellular domain. EMBO J 1997;16:4184–4193. [PubMed: 9250662]
55. Kaufmann N, Wills ZP, Van Vactor D. *Drosophila* Rac1 controls motor axon guidance. Development 1998;125:453–461. [PubMed: 9425140]
56. Ng J, Nardine T, Harms M, Tzu J, Goldstein A, Sun Y, Dietzl G, Dickson BJ, Luo L. Rac GTPases control axon growth, guidance and branching. Nature 2002;416:442–447. [PubMed: 11919635]
57. Grenningloh G, Rehm EJ, Goodman CS. Genetic analysis of growth cone guidance in *Drosophila*: fasciclin II functions as a neuronal recognition molecule. Cell 1991;67:45–57. [PubMed: 1913818]
58. Canal I, Ferrus A. The expression of Ultrabithorax (Ubx) during development of the nervous system of *Drosophila*. J Neurogenet 1987;4:161–177. [PubMed: 3112353]
59. Lengyel JA, Iwaki DD. It takes guts: the *Drosophila* hindgut as a model system for organogenesis. Dev Biol 2002;243:1–19. [PubMed: 11846473]
60. Ghabrial A, Luschnig S, Metzstein MM, Krasnow MA. Branching morphogenesis of the *Drosophila* tracheal system. Annu Rev Cell Dev Biol 2003;19:623–647. [PubMed: 14570584]
61. Ribeiro C, Petit V, Affolter M. Signaling systems, guided cell migration, and organogenesis: insights from genetic studies in *Drosophila*. Dev Biol 2003;260:1–8. [PubMed: 12885551]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

62. Uv A, Cantera R, Samakovlis C. *Drosophila* tracheal morphogenesis: intricate cellular solutions to basic plumbing problems. Trends Cell Biol 2003;13:301–309. [PubMed: 12791296]

63. Boube M, Martin-Bermudo MD, Brown NH, Casanova J. Specific tracheal migration is mediated by complementary expression of cell surface proteins. Genes Dev 2001;15:1554–1562. [PubMed: 11410535]

64. Glise B, Noselli S. Coupling of Jun amino-terminal kinase and Decapentaplegic signaling pathways in *Drosophila* morphogenesis. Genes Dev 1997;11:1738–1747. [PubMed: 9224722]

65. Bustelo XR. Understanding Rho/Rac biology in T-cells using animal models. BioEssays 2002;24:602–612. [PubMed: 12111721]

66. Doody GM, Billadeau DD, Clayton E, Hutchings A, Berland R, McAdam S, Leibson PJ, Turner M. Vav-2 controls NFAT-dependent transcription in B-but not T-lymphocytes. EMBO J 2000;19:6173–6184. [PubMed: 11080163]

67. Zakaria S, Gomez TS, Savoy DN, McAdam S, Turner M, Abraham RT, Billadeau DD. Differential regulation of TCR-mediated gene transcription by Vav family members. J Exp Med 2004;199:429–434. [PubMed: 14757747]

68. Newsome TP, Schmidt S, Dietzl G, Keleman K, Asling B, Debant A, Dickson BJ. Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in *Drosophila*. Cell 2000;101:283–294. [PubMed: 10847683]

69. Zhou K, Wang Y, Gorski JL, Nomura N, Collard J, Bokoch GM. Guanine nucleotide exchange factors regulate specificity of downstream signaling from Rac and Cdc42. J Biol Chem 1998;273:16782–16786. [PubMed: 9642235]

70. Buchsbaum RJ, Connolly BA, Feig LA. Regulation of p70 S6 kinase by complex formation between the Rac guanine nucleotide exchange factor (Rac-GEF) Tiam1 and the scaffold spinophilin. J Biol Chem 2003;278:18833–18841. [PubMed: 12531897]

71. Buchsbaum RJ, Connolly BA, Feig LA. Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogen-activated protein kinase cascade. Mol Cell Biol 2002;22:4073–4085. [PubMed: 12024021]

72. Bubeck Wardenburg J, Pappu R, Bu JY, Mayer B, Chernoff J, Straus D, Chan AC. Regulation of PAK activation and the T cell cytoskeleton by the linker protein SLP-76. Immunity 1998;9:607–616. [PubMed: 9846482]

73. King N, Hittinger CT, Carroll SB. Evolution of key cell signaling and adhesion protein families predates animal origins. Science 2003;301:361–363. [PubMed: 12869759]

74. Hibberd DJ. Observations on the ultrastructure of the choanoflagellate *Codosiga botrytis* (Ehr.) Saville-Kent with special reference to the flagellar apparatus. J Cell Sci 1975;17:191–219. [PubMed: 1089676]

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.yexcr.2005.04.035.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

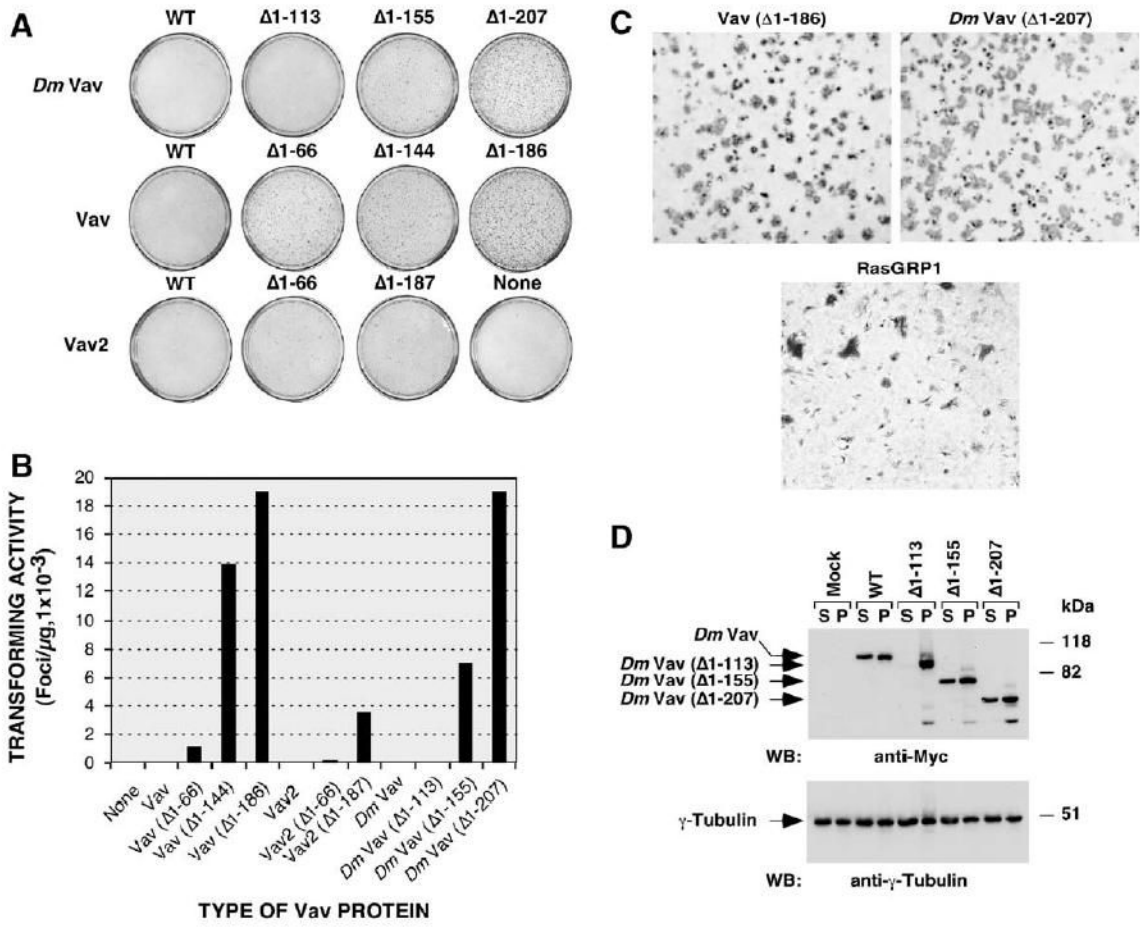


Fig. 1.

Transforming activity of *Dm*Vav proteins with amino acid deletions in the CH-Ac region. (A) Focus formation assays were conducted with plasmids encoding *Dm*Vav (upper row), mouse Vav (middle row), or mouse Vav2 (lower row) that were either wild type (WT) or deleted in the indicated N-terminal regions. NIH3T3 cells were transfected with vectors encoding either wild type versions (1 μg each) or mutant Vav proteins (0.1 μg each). As a control, we used cells transfected with high molecular weight calf thymus DNA alone (None). After transfection, cells were cultured for 15 days and stained with Giemsa to visualize the foci of transformed cells. (B) Transforming activity of Vav mutants. Foci obtained in the above transfections were counted de visu and numbers obtained represented in a bar histogram. Values were normalized considering the amount of plasmid DNA used in each transfection. (C) Morphology of the foci obtained with the indicated oncogenes. (D) Expression of the *Dm*Vav proteins used in these experiments. COS1 cells were transfected with either empty plasmid (Mock) or with expression vectors encoding the indicated *Dm*Vav proteins. After 48 h, total cell extracts were obtained and protein expression evaluated by anti-Myc immunoblots (upper panel). Equal loading of samples was demonstrated using anti-γ-tubulin antibodies (lower panel). The migration of molecular weigh markers is indicated on the right. The position of *Dm*Vav and γ-tubulin proteins is indicated by arrows on the left. S, supernatant fraction after the centrifugation of cellular extracts after cell lysis. P, pellet obtained after the centrifugation of the cellular lysates. WB, Western blot.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

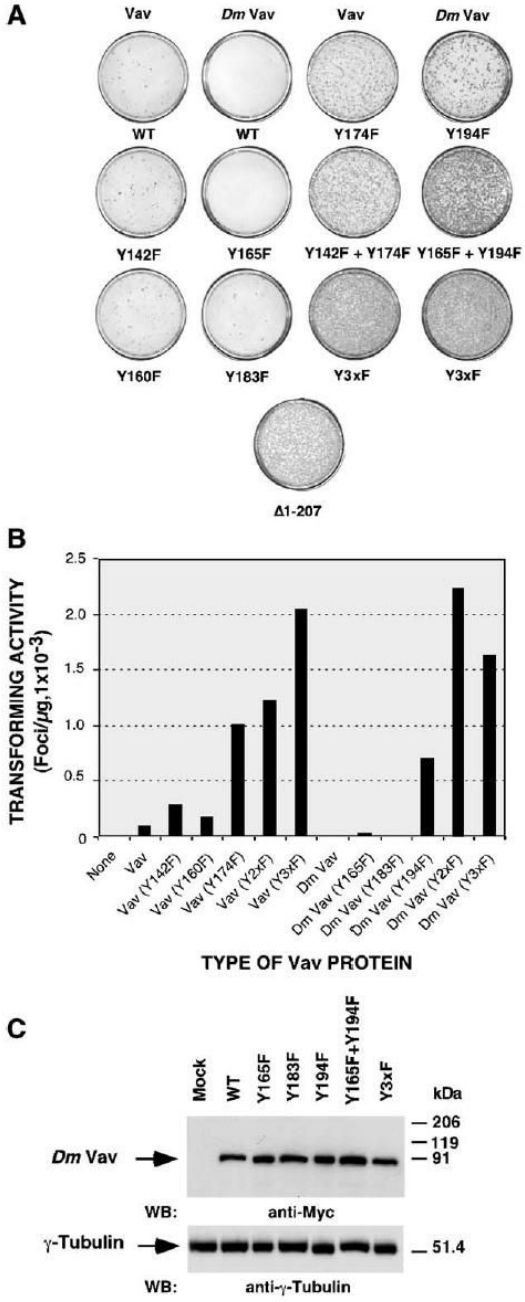


Fig. 2.

Transforming activity of *Dm*Vav proteins with Y to F mutations in the Ac region. (A) Focus formation assays were conducted with plasmids (1 μg each) encoding *Dm*Vav or mouse Vav that were either wild type (WT) or mutated in the indicated tyrosine residues. Y3xF is an abbreviation for the triple Vav Y142F + Y160F + Y174F and *Dm*Vav Y165F + Y183F + Y194F mutants. As a comparative control, we included transfections with a plasmid encoding *Dm*Vav (Δ1–207; 0.1 μg). After transfection, cells were cultured for 15 days and stained with Giemsa to visualize the foci of transformed cells. (B) Transforming activity of Vav mutants. Foci obtained in the above transfections were counted de visu and numbers represented in a bar histogram. Values were normalized considering the amount of plasmid DNA used in each

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

transfection. (C) Expression of the *Dm*Vav proteins used in these experiments. COS1 cells were transfected with either empty plasmid (Mock) or with expression vectors encoding the indicated *Dm*Vav proteins. After 48 h, total cell extracts were obtained and protein expression evaluated by anti-Myc immunoblots (upper panel). Equal loading of samples was demonstrated using anti-γ-tubulin antibodies (lower panel). The migration of molecular weigh markers is indicated on the right. The position of *Dm*Vav and γ-tubulin proteins is indicated by arrows on the left.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

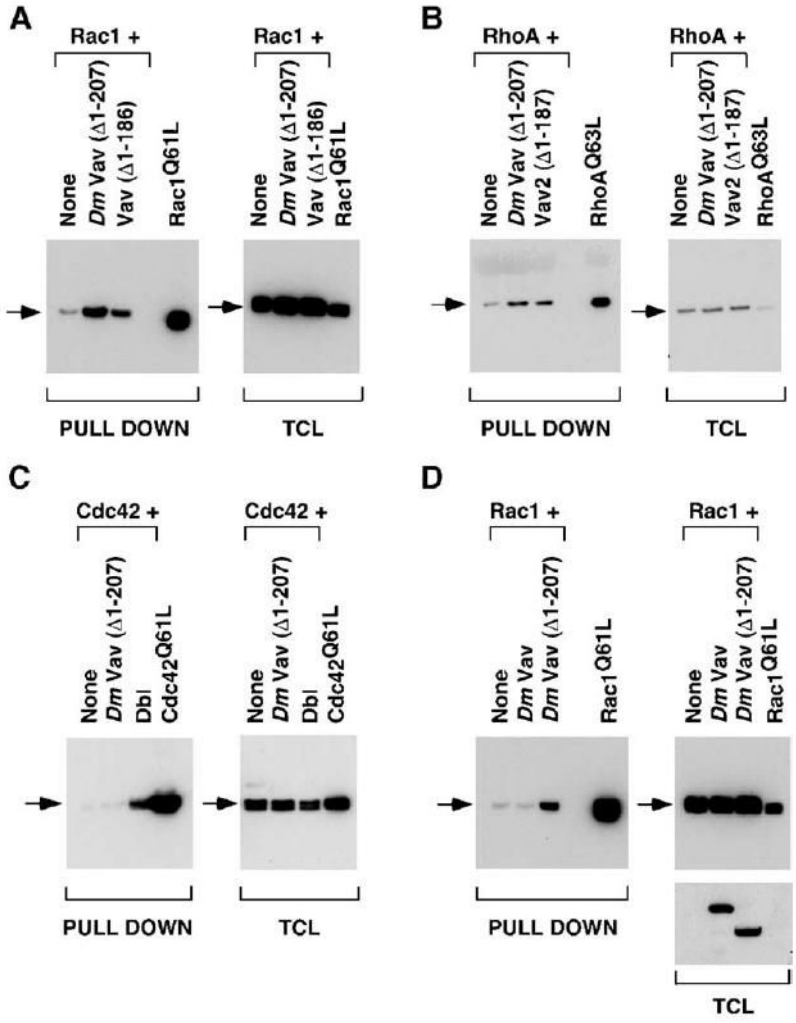


Fig. 3.

Activation of Rho/Rac GTPases by *Dm*Vav proteins. (A–D) COS1 cells were transiently transfected with the combination of proteins indicated at the top of each panel. After transfection, the GTP-bound levels of Rac1 (A, D), RhoA (B) and Cdc42 (C) were evaluated by pull down assays with specific GST bait proteins. (A–C) Left panels, result of the pull-down experiments using anti-AU5 immunoblots. Right panels, levels of expression of the GTPases in the lysates used for the pull-down experiments. (D) Left panel, result of the pull-down experiment using anti-AU5 immunoblots. Right panels, expression of Rac1 (upper panel) and *Dm*Vav proteins (lower panel) used in this study. The migration of the GTPases is indicated by arrows. TCL, total cellular lysate.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

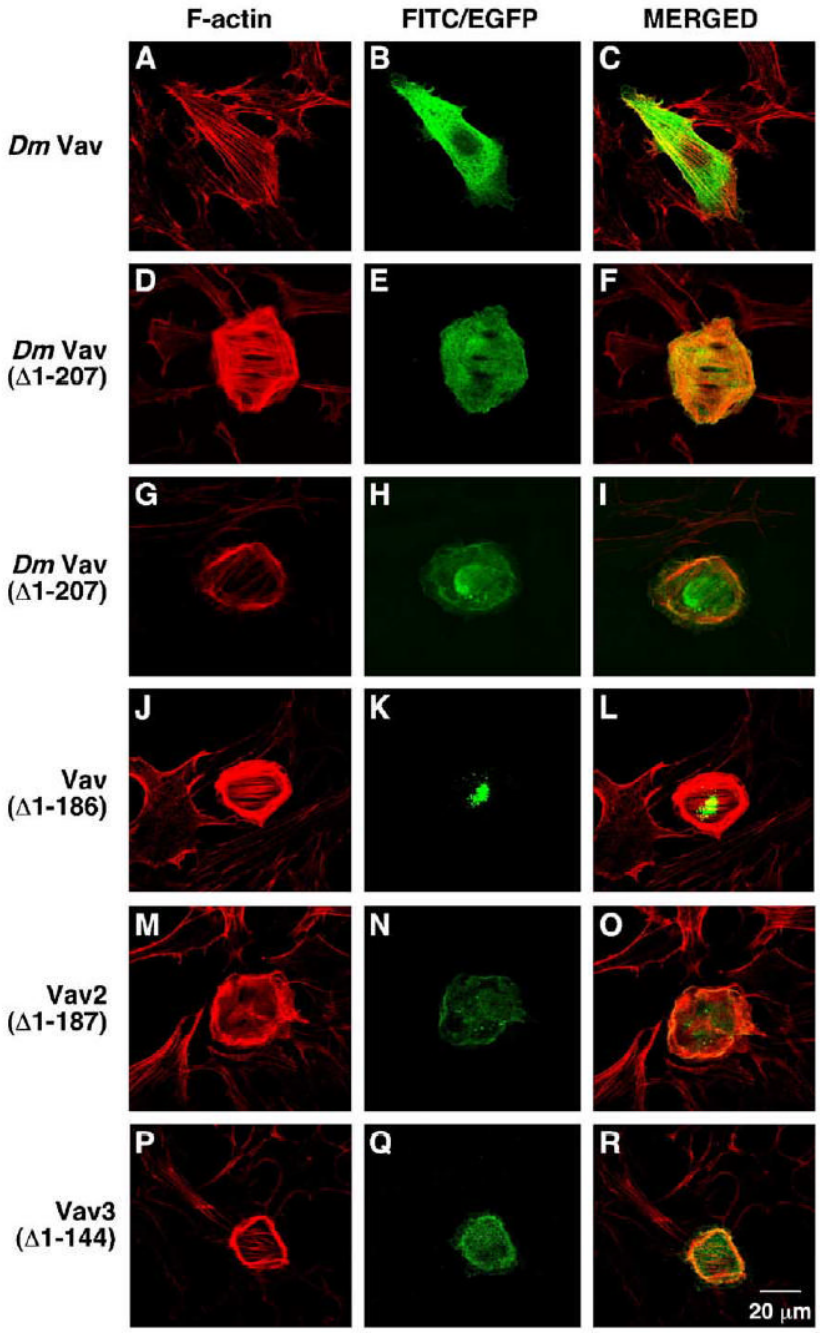


Fig. 4.

Morphological change induced by Vav family proteins in NIH3T3 cells. Cells were transfected with plasmids encoding the indicated Vav proteins (left side). After transfection, cells were stained with rhodamine-phalloidin and, in the case of *Dm*Vav-expressing cells (panels A–I), immunostained with anti-Myc antibodies followed by FITC labeled-secondary antibodies. Cells were then subjected to confocal immunofluorescence analysis. The localization of F-actin (panels A, D, G, J, M, P) and Vav proteins (panels B, E, H, K, N, Q) is shown in red and green, respectively. The areas of co-localization are shown in yellow (panels C, F, I, L, O, R).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

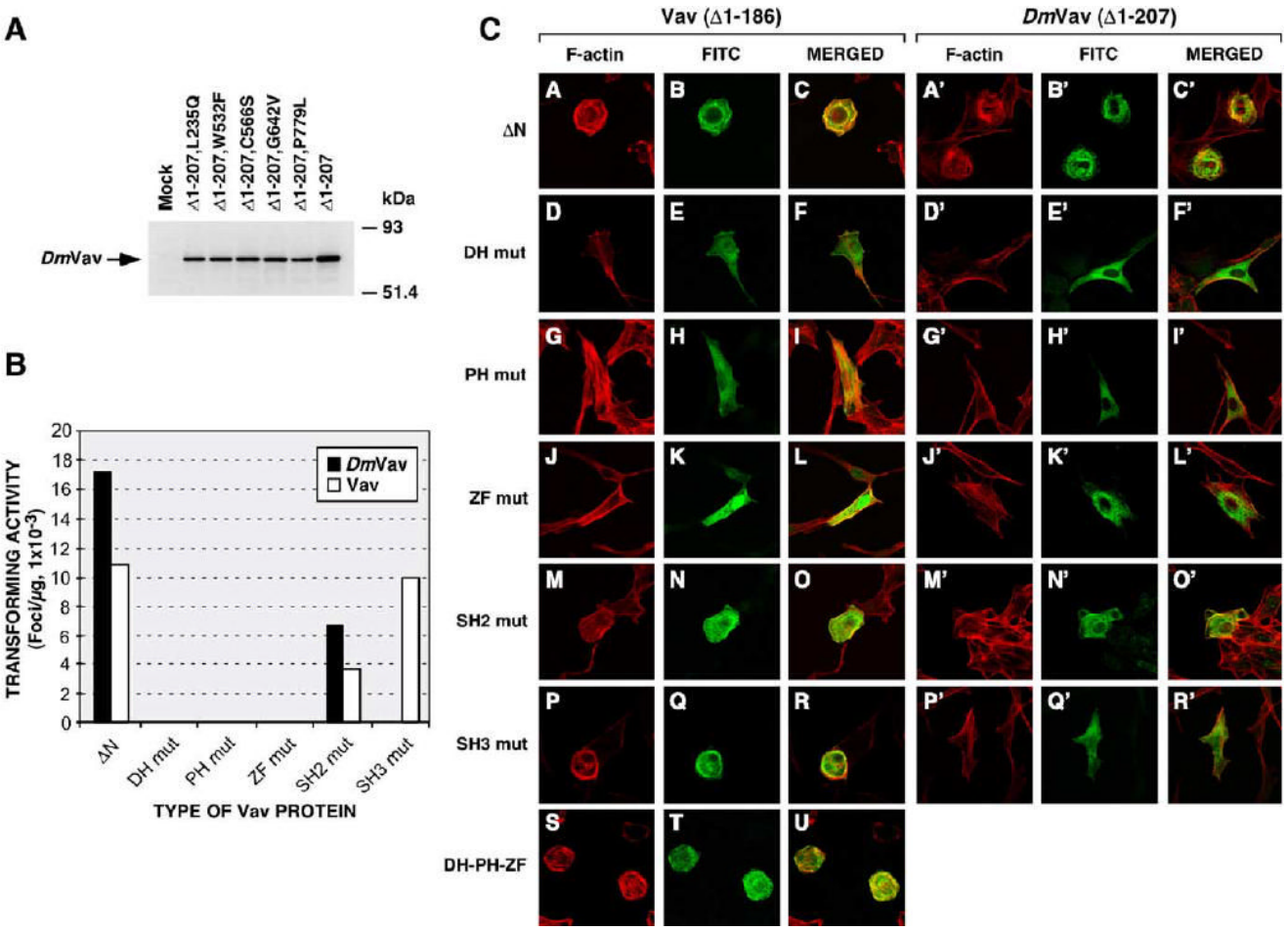


Fig. 5.

(A) Expression of the *Dm*Vav mutant proteins used in these experiments. Expression vectors encoding the indicated mutants of Myc-tagged *Dm*Vav (top) were transfected in COS1 cells. 24 h after transfection, total cell lysates were obtained and analyzed by immunoblot analysis with anti-Myc antibodies. The migration of *Dm*Vav proteins is indicated with an arrow. (B) Transforming activity of *Dm*Vav (Δ1–207) and mouse Vav (Δ1–186) proteins with inactivating point mutations in the DH, PH, ZF, SH2, and SH3 domains. Focus assays were performed as indicated in the legend to Fig. 1. ΔN, N-terminal deleted forms of *Dm*Vav and mouse Vav; mut, mutant. (C) Morphological change induced by *Dm*Vav and mouse Vav mutant proteins in NIH3T3 cells. Cells were transfected with plasmids encoding the indicated mutant proteins (left side) of mouse Vav (Δ1–186) (panels A–U) and *Dm*Vav (Δ1–207) (panels A′–R′). After transfection, cells were stained with rhodamine-phalloidin and with antibodies to the Vav DH region (panels A–U) or the Myc epitope (panels A′–R′). After incubation with FITC-labeled secondary antibodies, cells were subjected to confocal immunofluorescence analysis. The localization of F-actin and Vav proteins is shown in red and green, respectively. The areas of co-localization are shown in yellow. DH-PH-ZF (lower panel) refers to a truncated version of mouse Vav containing only the central DH-PH-ZF regions. The specific mutations used to inactivate each domains have been indicated in (A) and the main text.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

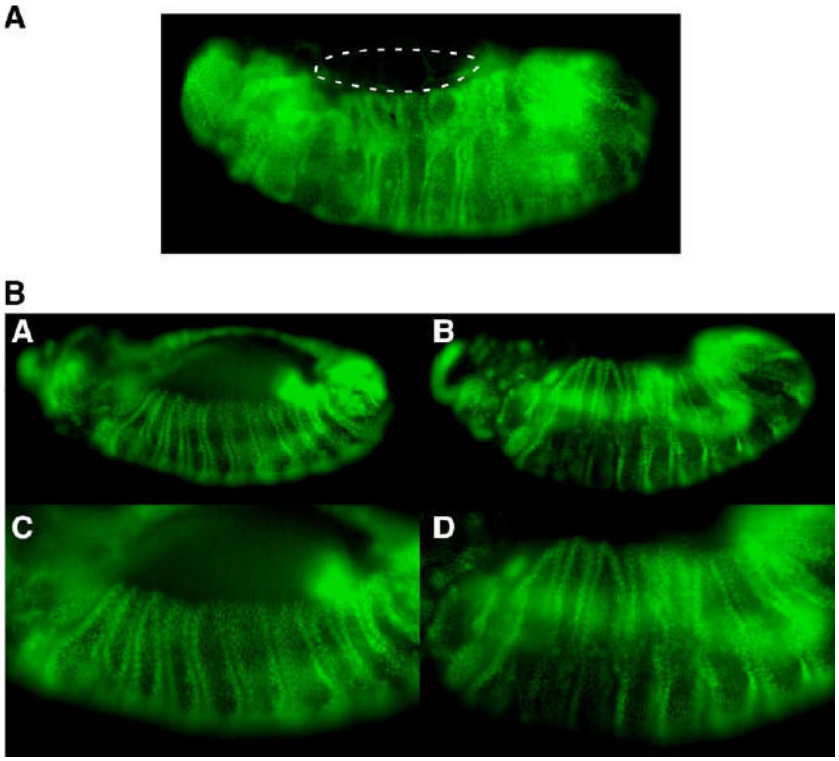


Fig. 6.

*Dm*Vav induces defects in embryonic dorsal closure. To show defects in the ectoderm, embryos were stained with an anti-fasciclin III antibody followed by a FITC-labeled secondary antibody. (A) Expression of *Dm*Vav (Δ1–207) results in a failure in dorsal closure. The limit of the unclosed area in the 16-stage embryo is indicated with a discontinuous white circumference. (B) Ectoderm histology of wild type (panels A and C) and *Dm*Vav (Δ1–207)-expressing embryos (panels B and D) at stage 13. Note that the distribution of fasciclin III in the cells of the leading edge in experimental embryos (B, D) is abnormal, being detected in the dorsal side of these cells from where it is excluded in wild type embryos (A, C). The magnification used was 20× (for panels A and B) and 40× (for panels C and D).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

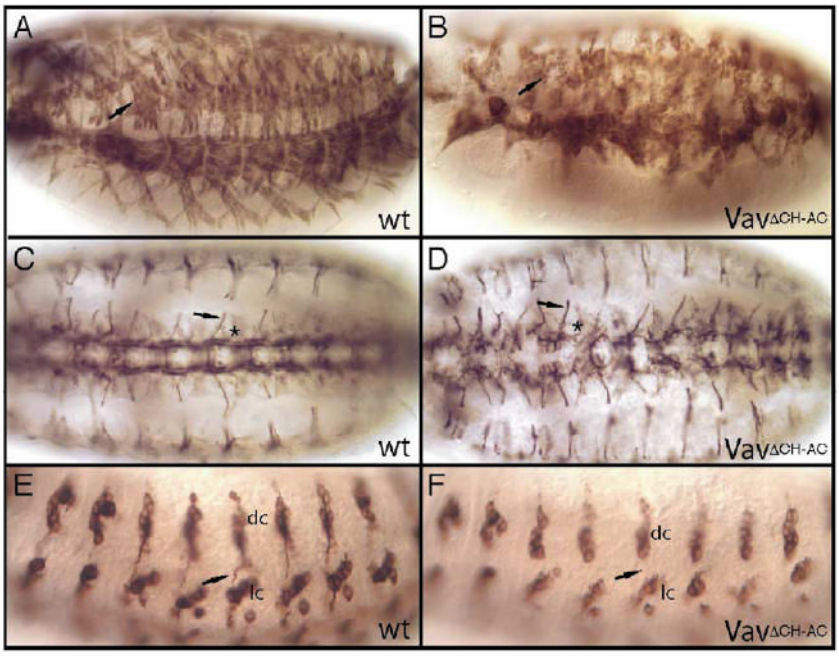


Fig. 7.

*Dm*Vav induces defects in myoblast fusion (A, B) as well as in the migration and guidance of axons of neurons from the central (C, D) and peripheral (E, F) nervous system. Wild type and *Dm*Vav (Δ1–207)-expressing embryos in specific cell types were stained using anti-muscle myosin (A, B), anti-fasciclin II (C, D), or 22C10 (E, F) antibodies. After immunostaining, embryos were mounted and photographed. (A, B) Arrow indicates unfused myoblasts in *Dm*Vav (Δ1–207)-expressing embryos (B). (C, D) Some longitudinal axons are missing in embryos expressing *Dm*Vav (Δ1–207) (asterisks) and axons look thicker than wild type (wt) ones (arrows). In addition, axons fail to extend between the dorsal (dc) and the lateral (lc) clusters of the PNS (arrows in E and F). wt, wild type; $Vav^{\Delta CH\text{-}AC}$, *Dm*Vav (Δ1–207) mutant.

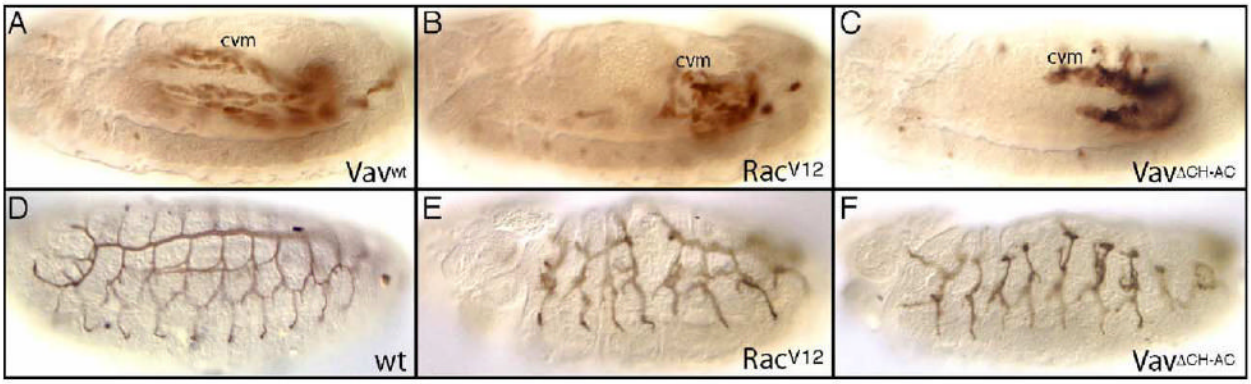


Fig. 8.

*Dm*Vav induces defects in the migration of caudal visceral mesodermal cells (cmv; A–C) and in tracheal development (D–F). Embryos expressing wild type *Dm*Vav (Vav^{wt}), *Dm*Vav (Δ1–207, $Vav^{\Delta CH\text{-}AC}$) or the constitutively active version of *Dm*Rac1 (Rac^{V12}) were stained using anti-HA (A–C) or 2A12 (D–F) antibodies, mounted, and photographed.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

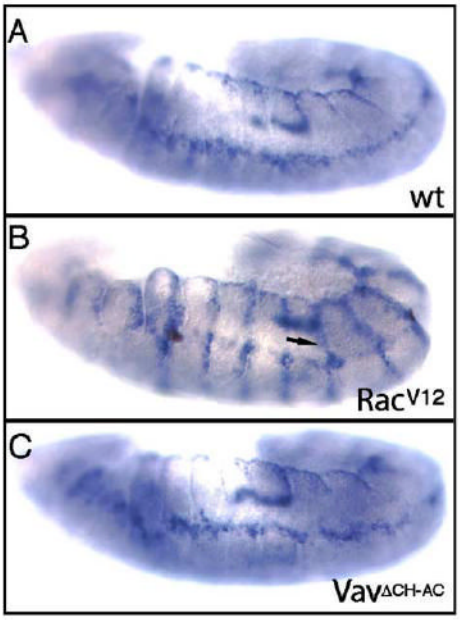


Fig. 9.

*Dm*Vav cannot connect Rac1 activation to the stimulation of the JNK pathway. Embryos wild type (wt, A) or expressing the indicated *Dm*Rac1 (B) and *Dm*Vav (C) proteins in segmental stripes of the embryonic ectoderm were subjected to whole mount in situ hybridization using an anti-sense probe to the JNK target *dpp*. After development of signals, embryos were mounted and photographed. The arrow indicates a stripe positive for *dpp* expression (B).